Integrating human and environmental health in antibiotic risk assessment: a critical analysis of protection goals, species sensitivity and antimicrobial resistance

Gareth Le Page[a], Lina Gunnarsson[a], Jason Snape[b, c], Charles R. Tyler[a]

[a]Biosciences, College of Life and Environmental Sciences, University of Exeter, Geoffrey Pope, Stocker Road, Exeter, Devon, EX4 4QD, UK.

[b]AstraZeneca, Global Environment, Alderley Park, Macclesfield, Cheshire, SK10 4TF, UK

[c]School of Life Sciences, Gibbet Hill Campus, The University of Warwick, Coventry, CV4 7AL

Corresponding author: Charles R Tyler. Geoffrey Pope, College of Life and Environmental Sciences, University of Exeter, Exeter, EX4 4QD. C.R.Tyler@exeter.ac.uk

Running title – Antibiotics: Linking human and environmental risk

Funding: This work was supported by the AstraZeneca Global SHE Research Programme

Competing financial interests declaration: GLP is a former employee and current shareholder of AstraZeneca PLC. JRS is an employee and shareholder of AstraZeneca PLC.

1 Abstract

Antibiotics are vital in the treatment of bacterial infectious diseases but when released into the environment they may impact non-target organisms that perform vital ecosystem services and enhance antimicrobial resistance development with significant consequences for human health. We evaluate whether the current environmental risk assessment regulatory guidance is protective of antibiotic impacts on the environment, protective of antimicrobial resistance, and propose science-based protection goals for antibiotic manufacturing discharges. A review and meta-analysis was conducted of aquatic ecotoxicity data for antibiotics and for minimum selective concentration data derived from clinically relevant bacteria. Relative species sensitivity was investigated applying general linear models, and predicted no effect concentrations were generated for toxicity to aquatic organisms and compared with predicted no effect concentrations for resistance development. Prokaryotes were most sensitive to antibiotics but the range of sensitivities spanned up to several orders of magnitude. We show reliance on one species of (cyano)bacteria and the 'activated sludge respiration inhibition test') is not sufficient to set protection levels for the environment. Individually, neither traditional aquatic predicted no effect concentrations nor predicted no effect concentrations suggested to safeguard for antimicrobial resistance, protect against environmental or human health effects (via antimicrobial resistance development). Including data from clinically relevant bacteria and also more species of environmentally relevant bacteria in the regulatory framework would help in defining safe discharge concentrations for antibiotics for patient use and manufacturing that would protect environmental and human health. It would also support ending unnecessary testing on metazoan species.

Keywords: Antibiotics; Environmental risk assessment; Antibiotic manufacturing; Antimicrobial resistance, Ecotoxicology, Pharmaceuticals

2 Highlights

- Bacteria are most sensitive to antibiotics but there is high interspecies variation
- ERA is not protective of environmental bacteria underpinning key ecosystem services
- ERA does not assess antimicrobial resistance
- Metazoans lack the drug target and never drive the ERA for antibiotics
- Antibiotic production discharge limit of 100ng/l in the mixing zone is recommended

3 Introduction:

Antibiotics are crucial in human healthcare. They are used in the treatment of bacterial infectious diseases, supporting surgical interventions, and in cancer and prophylactic treatment. Antibiotics are also used widely in livestock and domestic animal veterinary treatments and as growth promoters in aquaculture. Global production of antibiotics for human use is valued at $40 billion a year (O'Neill 2015) illustrating their societal and economic importance. Antibiotic consumption is on the rise and between the years 2000 and 2010 there was an estimated 36% increase in use globally for human healthcare (Van Boeckel et al. 2014).

Antibiotics, as other pharmaceuticals, enter the environment via patient and animal use, through manufacturing plants and/or improper disposal. Common points of entry into the environment from human therapeutic use are via effluents from hospitals, domestic sewerage treatment plants, as well as via leachates from landfill sites. Antibiotics can enter into surface waters from sewerage treatment plants directly or they can be transferred via surface run off. Ground waters can be exposed from agricultural land treated with sewage sludge biosolids as a source of fertiliser (Kümmerer 2009). Veterinary antibiotics enter the aquatic environment either directly, if treated animals are poorly managed and have access to surface water, or via groundwater from the manure of treated livestock (Davies 2012; Kümmerer 2009). Antibiotics in surface waters and sewerage treatment plant effluents/wastewaters are generally measured at concentrations ranging between 0.01 and 1.0 µg/L (Batt et al. 2007;

Miao et al. 2004; Monteiro and Boxall 2010; Watkinson et al. 2009). The highest levels of antibiotic residues in effluents - in the milligram per litre range, with records in excess of 1000 mg/L - are reported from manufacturing plants in China and India (Larsson 2014; Larsson et al. 2007; Li et al. 2008; O'Neill 2015). Hospital effluents too can contain antibiotic residues in the milligram per litre concentration range (Brown et al. 2006; Watkinson et al. 2009).

Antibiotics affect prokaryotic cells via a number of distinct mechanisms of action, including the inhibition of cell envelope synthesis, inhibition of protein synthesis or inhibition of nucleic acid (DNA/RNA) synthesis. Antibiotics are designed for use in the treatment of bacterial infection in humans and livestock and are thus developed to avoid, or limit, effects on mammalian cells. It is, therefore, reasonable to assume that environmental bacteria are more likely to be adversely affected as a result of non-therapeutic exposure compared with aquatic vertebrates, such as fish.

Within Europe, an environmental risk assessment (ERA) is required for a medicine if the predicted environmental concentration exceeds 10 ng/l (EMA 2006). In the USA effect studies are triggered if the expected environmental concentration exceeds 100 ng/L (US Food and Drug Administration 1998). The ERA aims to establish the safe concentrations for the protection of wildlife populations, ecosystem structure and function and includes the calculation of three predicted no effect concentrations (PNEC) for aquatic organisms, namely $PNEC_{surfacewater}$ ($PNEC_{SW}$), $PNEC_{microorganism,}$ and $PNEC_{groundwater}$ (EMA 2006). These are determined by establishing a no observed effect concentration (NOEC, the test concentration at which there is no statistically significant effect in the response being tested, such as on growth rate or reproduction) for a range of aquatic taxa and applying an assessment factor of ten to account for variability in species sensitivity and extrapolation from laboratory data to the field. $PNEC_{microorganism}$ is based on the 'activated sludge respiration inhibition test' (ASRIT, OECD 2010) and is primarily used to establish risk to microorganisms in (and the function of) sewerage treatment plants. The $PNEC_{groundwater}$ is based on a chronic test with *Daphnia magna* (e.g. OECD 211 test guideline, (OECD 2012))

and $PNEC_{SW}$ is calculated from the toxicity to three eukaryotic species – a green algae, invertebrate and fish. For antibiotics, in Europe the ERA guidance encourages ecotoxicity testing with prokaryotes rather than a green algae "*as they are [a] more sensitive indicator organisms than green algae*" (EMA 2006), and this is conducted in one species of cyanobacteria only.

There is concern that the ERA for antibiotics is biased towards testing on metazoan species (invertebrates and fish in this instance), and does not consider fully the possible impacts of antibiotics on microbial community structure, function and resilience (Agerstrand et al. 2015; Brandt et al. 2015). This is a major shortfall considering the fundamental ecosystem services microbial communities provide (e.g. primary production, nutrient cycling, metabolism and degradation of organic, inorganic and synthetic compounds). A major aim of this meta-analysis therefore was to test if current ERA is protective of vulnerable populations in the environment.

Microorganisms exposed to antibiotics at low, sub-lethal or sub-inhibitory exposure concentrations can develop, or acquire, antimicrobial resistance (AMR) and this has been identified as a major threat to public health (Smith and Coast 2002; World Health Organization 2014). AMR is likely to persist and disseminate in diverse environments, including in aquatic ecosystems (Laxminarayan et al. 2013; Taylor et al. 2011). Where the benefit of possessing and expressing the resistance gene outweighs the fitness costs of carriage, antibiotics in the environment may select for and enrich resistance genes in bacterial populations/communities which can then harbour these resistance determinants and transfer them to human pathogens (Ashbolt et al. 2013).

To ensure clinical efficacy and protection of human health, minimum inhibitory (growth) concentrations (MICs, the lowest concentration at which there is no observable growth) are monitored in clinically relevant bacteria (CRB) and recorded in the European Committee on Antimicrobial Susceptibility Testing database (http://www.eucast.org). In addition to monitoring MICs in clinically relevant species, studies with clinical isolates have also identified the lowest

concentration that will select for AMR, called minimum selective concentrations (MSCs). MSCs are the minimum concentration at which the presence and expression of resistance gene(s) give bacteria a fitness advantage over non-resistant cells of the same species/strain. This can occur at concentrations considerably below the MIC of the non-resistant cells (Gullberg et al. 2011). Indeed, selection may occur at exposures up to two orders of magnitude lower than the MIC for growth (Gullberg et al. 2011; Hughes and Andersson 2012; Lundström et al. 2016).

From both human and environmental health perspectives, it is important that risk assessment frameworks incorporate the risk of AMR selection. An approach to establish a surrogate PNEC for AMR ($PNEC_R$) has been suggested adopting MICs from CRB, which are available through the European Committee on Antimicrobial Susceptibility Testing database (Bengtsson-Palme and Larsson 2016). This is the most comprehensive dataset available where theoretical PNECs ($PNEC_{R(T)}$) have been calculated for 111 antibiotics. This approach uses growth (via the MIC) to predict upper boundaries for resistance, although there has been no verification of an increase in resistance determinants. The approach also assumes that the CRB are representative of the diversity of bacteria in nature. Furthermore, whilst AMR maybe enriched at concentrations well below the MIC of clinical bacteria, the AMR enrichment could potentially occur at concentrations below the effects determined in traditional ERA ecotoxicity growth tests on cyanobacteria. This meta-analysis therefore also sought to determine the relationship between protection goals proposed to protect against resistance development and the traditional aquatic protection goals; i.e. establish if the proposed methods used to derive a PNEC for AMR development ($PNEC_R$) are protective of those currently used for aquatic ecosystem function ($PNEC_{sw}$) and *vice versa*.

Recognising that antibiotic releases from drug production and formulation facilities represent 'hot spots' for the development of AMR it is critical that these discharges are minimised and managed effectively across the whole supply chain. To address this concern, the pharmaceutical industry recently established

an AMR Road map which included a commitment to "establish science-driven, risk-based targets for discharge concentrations for antibiotics and good practice methods to reduce environmental impact of manufacturing discharges, by 2020" {IFPMA, 2016 #415}.

To improve the testing paradigm for antibiotics for use in prospective regulatory frameworks and to establish safe discharge concentrations for antibiotic production, we conducted a meta-analysis based on a systematic review of the publically available aquatic ecotoxicity data and clinically relevant MICs for antibiotics. Specifically we; 1) assess the relative sensitivity of commonly used taxa in aquatic ecotoxicity, with a MOA perspective, to evaluate the reliability of the current ERA of antibiotics to identify risk to vulnerable populations; 2) assess the value of extending the toxicity testing for bacteria through an assessment on the relative sensitivity of several cyanobacterial species, the marine bacteria *Vibrio fischeri* and the CRB MICs; 3) critically evaluate the current proposed approaches for determining the risk of AMR and its incorporation into risk assessment for the protection of human health; i.e. whether a $PNEC_R$ is more or less protective than $PNEC_{SW}$ calculated using traditional ecotoxicity testing; 4) test the assumption that CRB adequately represent environmental bacteria and evaluate the use of pre-clinical MIC data for the protection of other bacterial species through a comparison of the NOECs for cyanobacteria with the adjusted MIC, calculated by Bengtsson-Palme and Larsson (2016) from CRB and; 5) use the empirical data collected in these analysis to help establish science-driven, risk-based targets for manufacturing discharge concentrations for antibiotics.

4 Methods

4.1 Data search strategy

A comprehensive literature search was carried out to identify studies reporting toxicological effects of antibiotics on aquatic taxa commonly used in ERA. These taxa included cyanobacteria, green algae, macrophytes (the latter currently used in ERA for agrochemicals, but not pharmaceuticals), invertebrates and fish. Data

were also collected for the effects of antibiotics on *Vibro fischeri*, for the ASRIT test and *Pseudomonas putida* (where available). Data were used in our analyses only if they met the following criteria: 1) the endpoint calculated was a NOEC, 50% effective concentration (EC50) or 50% inhibition concentration (IC50), the concentration at which 50% of the population are effected or inhibited respectively; 2) the methodology adopted was according to (or with minor deviations from) currently accepted regulatory protocols (e.g. Organisation for Economic Co-operation and Development (OECD) or International Organisation for Standardisation (ISO) test guidelines); 3) the aquatic species belong to the taxa described above; 4) exposures were for single species not multiple species/community exposures (with exception of the ASRIT which is a community based exposure) and; 5) organisms were exposed to a single antibiotic (not a chemical mixture).

The aim of this paper was to conduct a meta-analysis of available data in the context of current regulatory guidance that uses population-relevant endpoints to establish PNECs. Therefore NOECs and EC/IC50s for growth, reproduction or mortality only (or accepted surrogates e.g luminescence in *V. fischeri* or respiration in the ASRIT) were collected and analysed. Moreover, interpretation of biomarker endpoints in relation to population-based NOECs and EC/IC50s are not well established.

Searches and data collections were conducted for the following public databases and literature:

- Environmental data on antibiotics from the trade organisation for the research-based pharmaceutical industry in Sweden (LIF)), obtained from the Swedish fass.se database (www.fass.se accessed Jan 2016).
- Environmental data for antibiotics from the 'European public assessment report' database (www.ema.europa.eu, accessed Jan 2016).
- All published data in the Wikipharma database (http://www.wikipharma.org, accessed Jan 2016).

- All relevant data in the study by Vestel et al. (2015) which included the antibiotics azithromycin, bedaquiline, ceftobiprole, doripenem, linezolid, meropenem, sulfamethoxazole and trimethoprim.
- Data for sulfadiazine, neomycin and gentamycin, kindly provided by Merck Sharp & Dohme (MSD) through the 'Innovative Medicines Initiative' iPIE project (https://www.imi.europa.eu/content/ipie).
- A GoogleScholar search focused on cyanobacteria with the following search criteria for the 111 antibiotics listed in the paper by Bengtsson-Palme and Larsson (2016): *Antibiotic* cyanobacteria "OECD 201" OR "ISO8962" OR "ISO 8962" OR "850.4500" OR "E1440-91"
- The theoretical $PNEC_R$ ($PNEC_{R(T)}$) and the size-adjusted MIC (MIC_{aj}) for antibiotics were collected from Bengtsson-Palme and Larsson (2016). For antibiotics where less than 40 species have been tested in the European Committee on Antimicrobial Susceptibility Testing database, Bengtsson-Palme and Larsson (2016) calculated a size-adjusted MIC. This is a theoretical adjustment to the MIC to include 99% of CRB. The number derived from that calculation was rounded down to the nearest concentration in the range operated in the European Committee on Antimicrobial Susceptibility Testing protocol. $PNEC_{R(T)s}$ were calculated by applying an assessment factor of 10 to account for differences between inhibitory concentrations and selective concentrations of the antibiotics. Experimentally derived MSCs were identified from literature following a GoogleScholar search with search criteria: "Minimum selective concentration" MSC AND "antibiotic resistance". We highlight here that currently there is no internationally standardised test method for MSC and that extrapolation to the environment is poorly understood due to the complex nature of resistance enrichment, the complex nature of communities and a range of environmental factors that may influence the MSC (Khan et al. 2017; Quinlan et al. 2011).
- Antifungal and antiviral drugs obtained through our search criteria were excluded from this assessment.

All data derived from these searches are provided in the supplemental material, Table S1 and a flowchart to illustrate the data collection and statistical processes for these analyses is provided in figure S1.

4.2 Assessment of data reliability

Assessments on data reliability were undertaken using the 'Criteria for reporting and evaluating ecotoxicity data' (CRED) system that is specifically designed for the evaluation of ecotoxicity data for regulatory use (Moermond et al. 2016). In this system reliability is defined as "the inherent quality of a test report or publication relating to (preferably) standardized methodology and the way the experimental procedure and results are described to give evidence of the clarity and plausibility of the findings". The CRED system categorises the reliability of studies into one of four scores; R1 (reliable without constraints), R2 (reliable with constraints), R3 (unreliable) or R4 (not assignable). Studies identified as R3 are considered unsuitable for use in regulatory decision-making; whereas caution needs to be applied on a study-by-study basis for studies categorised as R2 or R4. The CRED evaluation method also provides guidance on the evaluation of the relevance of data (Moermond et al. 2016). This, however, was not applied as the data were considered relevant for this meta-analysis having fulfilled the selection criteria outlined in section 2.1. The CRED reliability score for each study is given in Table S1.

4.3 Relative taxa sensitivity data

The lowest 'reliable' NOEC and EC50 for each taxa were identified for each antibiotic. Data from studies that had CRED reliability scores of R1 and R2 were prioritised, without bias between R1 and R2, over those in the categories of R3 or R4. R4 data were selected over R3 data as the majority of R4 studies were assigned R4 due to unpublished/missing information in an otherwise (apparently) reliable study compared with R3, which were assigned unreliable for defined reason. The lowest 'reliable' NOEC and EC50 were applied in the analysis of relative taxa sensitivity and are presented in the Table S2. This conservative approach was deemed more appropriate rather than taking an average of all available data that has imbalanced taxa representation and varying data reliability.

An analysis of the relative sensitivity of cyanobacterial species adopted the same CRED criteria as described above to establish the lowest 'reliable' EC50. EC50s were used rather than NOECs as there was a larger dataset for cyanobacterial EC50s. These data are presented in Table S3.

4.4 Censored data

For some antibiotics the data was either left or right censored, meaning that the value was not a precise number and was given as greater than (>) or less than (<) the value reported (i.e. no effect at the highest test concentration or an observed effect at the lowest tested concentration, respectively). Censored data values were used when no other data were available (> than numbers would represent conservative values and < numbers were included only when they represented the lowest 'reliable' data value). Where data were censored, this is indicated in Table S1.

4.5 Establishing relative taxa sensitivity to antibiotics

A sensitivity ratio (SR) was calculated between the different taxa and cyanobacteria for each antibiotic, where data were available. The SR was calculated using the lowest NOEC (or NOEC and MIC_{aj} in the case of CRB) or EC50 using the following equation:

$$Log_{10}SR = logE_{cyanobacteria} - logE_{taxa}$$

where E is the endpoint (NOEC, EC50 or MIC_{aj}).

A SR >0 indicates that the cyanobacteria are more sensitive than the other taxa and less sensitive when SR <0. Each unit of SR is equivalent to an order of magnitude difference in sensitivity.

The difference between a SR calculated from NOECs compared with those calculated from EC50s was examined to identify how the endpoint used might impact the sensitivity ratio. Briefly, a generalised linear model (GLM) (Gaussian error family with identity link function) was constructed using the 'lmer' package with the restricted maximum likelihood method (Bates et al. 2015) in R

335 (version 3.3.0; R Project for Statistical Computing, Vienna, Austria). The model
336 residuals were normally distributed and significant differences identified using
337 the "lmerTest" package in R (Kuznetsova et al. 2013). SRs were used only where
338 a NOEC and EC50 were from the same species and publication in order to
339 exclude effects of different methodologies. The SRs calculated from EC50s were
340 significantly higher by 0.5 (p = 0.05) than those calculated from NOECs i.e.
341 cyanobacteria were less sensitive as measured by EC50s. As such, SRs calculated
342 from EC50s were only included in subsequent analyses comparing taxa
343 sensitivities where NOEC SRs were not available. We acknowledge that this will
344 have a small effect on the output of the models. However, because of the sparse
345 dataset and the relatively small difference in SR between EC50s and NOECs
346 compared with the differences between taxa, the inclusion of the EC50 SRs
347 where NOEC SRs are not available increases the number of SRs for comparison
348 and robustness of the models.
349
350 We established a GLM in R (version 3.3.0; R Project for Statistical Computing,
351 Vienna, Austria) to determine the effects of exposure duration on the EC50 for
352 *V. fischeri,* as EC50 are often reported for 5, 15 and 30 minutes and for 24 hours.
353 Censored data were removed and the remaining EC50s were $\log_{10}$ transformed
354 before use in the GLM (Gaussian error family with inverse link function) that was
355 constructed as described for comparing NOEC and EC50 SRs above. Significant
356 differences were identified by applying a TukeyHSD post hoc test. Twenty four
357 hour EC50s were significantly lower (p = <0.001) than those following shorter
358 exposure periods and data for this time point only were therefore used in
359 subsequent analyses on relative taxa sensitivities.
360
361 Differences in SR across all taxa for all antibiotics were analysed using a GLM.
362 The aim of the analysis was to compare the sensitivity of all taxa to
363 cyanobacteria. Cyanobacteria were chosen as the comparator because they are
364 assumed to be the most mode-of-action relevant taxa (therefore, most sensitive
365 species) in current ERA, and thus expected to drive the $PNEC_{SW}$. Briefly, to
366 assess for statistical differences in SR the GLM was constructed forcing the
367 intercept through 0 (the SR value of cyanobacteria). Therefore, the statistical

differences identified by "lmerTest" (Bates et al. 2015) represent the statistical difference from 0 and thus the statistical difference between the taxa and cyanobacteria. This allowed for the exclusion of cyanobacterial SRs in the GLM as the sensitivity of cyanobacteria were already accounted for in the calculation of the SRs. TukeyHSD post hoc tests were applied to identify any further differences between the taxa groups. Details on model construction and validation are provided in the Supplemental Material. Adopting the same process and validation steps, further GLMs were established for analyses of antibiotics with different mechanisms of actions and, where sufficient data were available, for antibiotic classes (a more detailed methodology for this is presented in Supplementary Material).

Antibiotics were classified into three groups based on their broad mode of action, specifically, cell envelope inhibitors (Anatomical Therapeutic Chemical (ATC) classification system codes J01C and J01D), Nucleic acid synthesis inhibitors (ATC codes J01E and J01M) and protein synthesis inhibitors (ATC codes J01A, J01B, J01F, J01G, J01XC, J01XX08, J01XX11 and QJ01XQ).

It is important to note that in addition to comparing different endpoints and methodologies, representation of antibiotics - in both potency and number of antibiotics with data - varied between and within taxa and antibiotic classes. We acknowledge this may introduce some uncertainty and potential bias in our analysis and have thus avoided the use of more complex model designs that might otherwise have introduced random factors and interactions. However, the biases mentioned above are unlikely to have an impact on the overall conclusions drawn from these analyses.

4.6 Calculation of PNECs

Where a full set of ecotoxicity data for an European Medicines Agency Phase 2 ERA was available (cyanobacteria, invertebrate and fish tests) a $PNEC_{SW}$ was calculated by taking the lowest NOEC of the three studies and applying an assessment factor of 10, as described in the regulatory guidance (EMA 2006). A theoretical $PNEC_R$ ($PNEC_{R(T)}$) was taken directly from (Bengtsson-Palme and

Larsson 2016). An experimental $PNEC_R$ ($PNEC_{R(Exp)}$) was calculated from the lowest experimental selective concentration and applying an assessment factor of 10.

There was not enough data to conduct species sensitivity distribution analysis and calculate 95% percentile protective limits, as this requires a minimum of 10 species and preferably more than 15 (ECHA 2008).

4.7 5th percentile determination

The calculated 5th percentiles for the NOEC and MIC data subsets were not normally distributed or fitting to other known distributions (e.g. gamma and weibull) before or following transformations (log, log_{10} or boxcox). The 5th percentile therefore was established using the non-parametric Harrell-Davis quantile estimator method. Analysis was conducted in R (version 3.3.0; R Project for Statistical Computing, Vienna, Austria) using the hdquantile function in the 'Hmisc' package (Harrell Jr 2016).

5 Results

Ecotoxicity data were collected for 79 antibiotics (Table S1) representing 48% of the 164 approved antibiotics identified in www.drugbank.ca and (Santos et al. 2017). Information on the ecotoxicity in cyanobacteria was available for 41 of these 79 antibiotics, but with NOECs for only 27 (16%). Antibiotics with NOECs for cyanobacteria were well distributed across all ATC sub-classes under J01, with exception of J01XX ('other antibacterials'; Figure S2).

A complete Phase 2, ERA dataset that included the full range of taxa for calculating a $PNEC_{SW}$ (EMA 2006) was available for only seven of these antibiotics. This may reflect the lack of pharmaceutical ERA datasets placed in the public domain and/or that few antibiotics have been approved since the existing European Medicines Agency guideline came into force in 2006 requiring full chronic toxicity testing on cyanobacteria/microalgae, invertebrates and fish and consequently lack a full ecotoxicity data set.

5.1 Relative species sensitivities

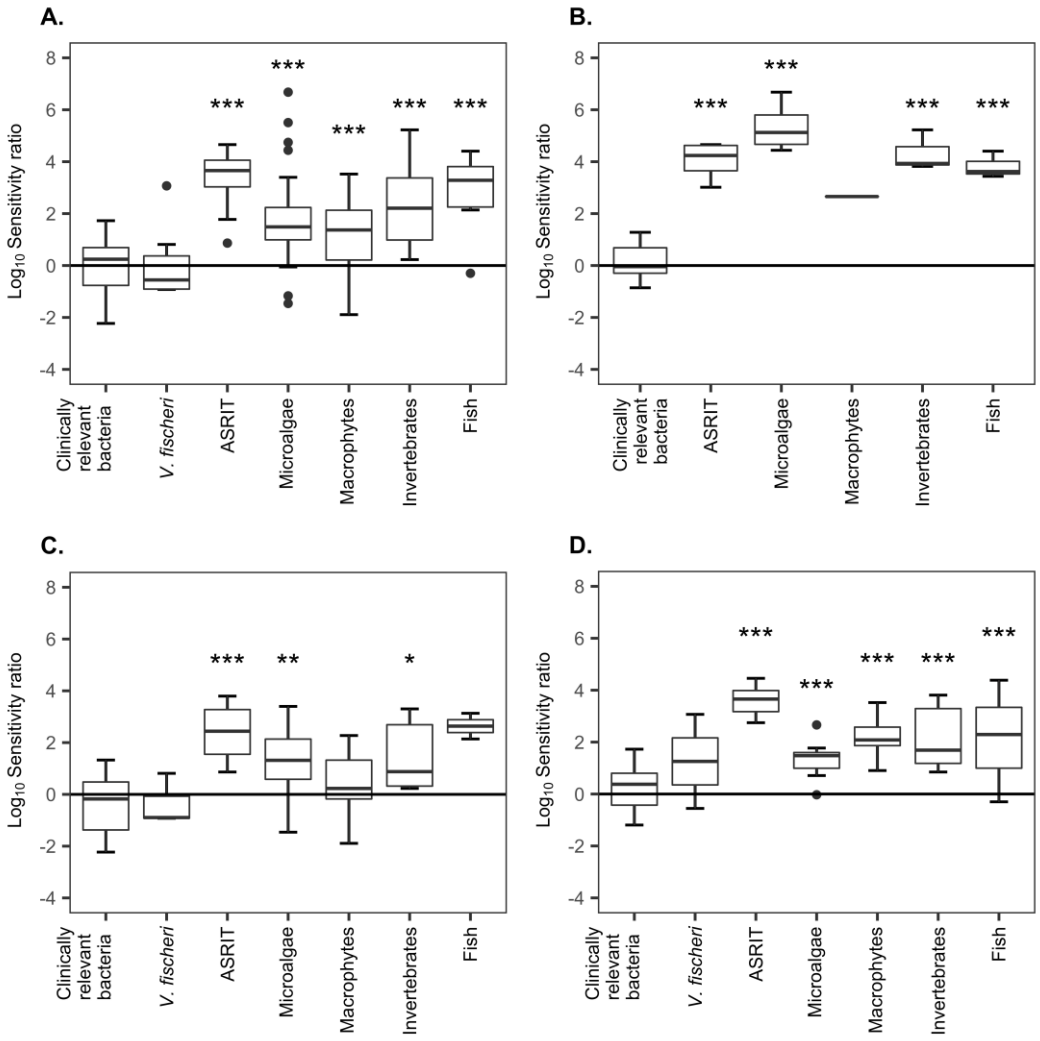

Figure 1. Boxplots of Log_{10} sensitivity ratio (SR) between cyanobacteria and other species/phyla for A) all antibiotics (n=37), B) cell envelope inhibitors (n=8), C) Nucleic acid synthesis inhibitors (n=12) and D) protein synthesis inhibitors (n=16). SR calculated based on log_{10}cyanobacteria NOEC or EC50 – log_{10}taxa NOEC or EC50. Where SR = 0 the sensitivity of the taxa is equal to cyanobacteria, represented by horizontal line, where SR >0 taxa had a lower sensitivity and <0 indicates higher comparative taxa sensitivity. Significant differences of SR from cyanobacteria in the generalised linear mixed models are indicated by: * p<0.05; ** p<0.01; *** *p*<0.001. Statistical tests were not performed on macrophytes in cell envelope inhibitors as there was only one antibiotic tested in macrophytes.

Overall, cyanobacteria were the most sensitive taxa of those currently recommended in the ERA of human pharmaceuticals (EMA 2006; US Food and Drug Administration 1998) (p = <0.001, Figure 1A) and they were equally sensitive as other bacteria (CRB and *V. fischeri*) and more sensitive than macrophytes (that are not currently required in ERA of pharmaceuticals; p = <0.001).

Figure 2. Chronic exposure effects of antibiotics on A) environmental bacteria and clinically relevant bacteria (no observed effect concentrations (NOEC) and adjusted minimum inhibitory concentrations respectively) and B) environmental bacteria 50% effective concentrations.

The sensitivity of cyanobacteria and CRB were not significantly different for any of the three broad antibiotic mechanisms of actions (Figures 1B-D); NOECs in cyanobacteria were lower than CRB MIC_{aj} for half (12 out of 24 antibiotics; Figure 2A). If we were to adopt the lowest MIC, instead of the modelled $MIC_{aj,}$ in this meta-analysis there would be more cases (18, rather than 12, out of 24) where the cyanobacteria were the most sensitive. Although there was no clear

relationship between the CRB MIC_{aj} and cyanobacterial NOECs the difference in sensitivity was up to two orders of magnitude for specific individual antibiotics (Figure 2A and 6C).

There were no significant differences in sensitivity to DNA or protein synthesis inhibiting antibiotics between *V. fischeri* and cyanobacteria (Figure 1; there were no data for cell-envelope inhibiting antibiotics). Of the seven antibiotics where SRs could be determined five were for quinolones giving an antibiotic class bias for the *V. fischeri* data. EC50s for *V. fischeri* were lower than those for the cyanobacteria on six occasions (Figure 2B), three of these were almost an order of magnitude lower (flumequine, lomefloxacin and oxolinic acid). *V. fischeri* was also the most sensitive organism to olfoxacin, with a NOEC one order of magnitude lower than the CRB MIC_{aj} (Figure 2A) and an EC50 half that for the cyanobacteria (Figure S3).

Pseudomonas putida, a model (soil) gram-negative bacteria used in standard growth inhibition test guideline (ISO 1995) was more sensitive than cyanobacteria for one out of five antibiotics (meropenem; Figure 2A and B).

The ASRIT (OECD 2010) was consistently between two and four orders of magnitude less sensitive than cyanobacteria, with the exception of trimethoprim (Figures 1 and 2 $p = <0.001$).

Figure 3. Chronic exposure effects (EC50s) of antibiotics on different cyanobacteria species.

There were large differences in sensitivity between cyanobacterial genera and species, with between two and three orders of magnitude difference in EC50s for 10 out of the 16 antibiotics, and approximately five orders of magnitude difference in response to the β-lactams amoxicillin and ampicillin (Figure 3). Overall, *Microcystis aeruginosa* was the most sensitive species (in half of the 16 antibiotics). *Anabaena cylindrical*, *Synechococcus leopoliensis* and *Microcystis wesenbergii* were each the most sensitive cyanobacterium for 2 of 16 antibiotics for which there were data on multiple species. *A. flos-aquae,* one of the cyanobacterial species recommended for testing in the OECD 201 test guideline, was the most sensitive species for only 1 of the 13 antibiotics in which it was tested. When considering antibiotic sensitivity based on their mechanisms of action, *Microcystis* species appeared to be more sensitive to nucleic acid

synthesis inhibitors (7 out of 9 antibiotics). *Microcystis* and *Synechococcus* species were the most sensitive to cell envelope inhibiting antibiotics. *Anabaena* genera were the most sensitive to the protein synthesis inhibitors (3 out of 6) and in two cases by more than an order of magnitude.

Overall, macrophytes were generally less sensitive to antibiotics compared with cyanobacteria with a wide range of SRs (Figure 1, p = <0.001). However, they showed equal sensitivity with cyanobacteria to nucleic acid synthesis inhibitors (average SR = 0.42; p = 0.3). The NOECs for trimethoprim and sulfadimethoxine were lower for macrophytes than for cyanobacteria (Figure 4A). A comparison of macrophyte and environmental bacteria EC50s is provided in Figure S3.

Figure 4. Chronic exposure effects of antibiotics on cyanobacteria and clinically relevant bacteria (no observed effect concentrations (NOEC) and adjusted minimum inhibitory concentrations respectively) compared with A) NOECs for microalgae and macrophytes and B) NOECs in invertebrates and fish.

Microalgae were also generally less sensitive to antibiotics than cyanobacteria (Figure 1, p = <0.001). However, for sulfadiazine and sulfadimethoxine the NOECs in microalgae (0.135 and 0.529 mg/L, respectively) were over an order of magnitude lower than for the lowest in the cyanobacteria (Figure 4A). We interpret these data with caution, however, as the results for the cyanobacteria

were derived from a study based on nominal (i.e. not measured) test exposure concentrations (Ando et al. 2007). A comparison of the EC50s for microalgae with environmental bacteria is shown in Figure S3.

Metazoans (fish and invertebrates) were significantly less sensitive across all antibiotics compared with cyanobacteria and often by between two and four orders of magnitude (with exception of tedlizolid phosphate, Figure 1 and 4, $p = < 0.001$, for both fish and invertebrates). There was substantial variation in SR between cyanobacteria and the metazoan taxa (as illustrated by the standard errors in the data; Figure 1). In the case of tedlizoid phosphate, a pro-drug, fish appeared more sensitive than cyanobacteria (NOECs of 0.032 versus 0.063 mg/L, respectively; Figure 4B). A MIC_{aj} for tedozolid (the active pharmaceutical ingredient) was not available from the Bengtsson-Palme and Larsson (2016) study, but a MIC of 0.016 mg/L (based on 12 species), corresponding to a MIC_{aj} <0.008 mg/L was recently (January 2017) reported the European Committee on Antimicrobial Susceptibility Testing database. This suggests that CRB are substantially more sensitive to tedozolid compared with fish and cyanobacteria. The fact that tedizolid phosphate (pro-drug) requires activation by phosphatases in the blood to convert it into the active ingredient (tedizolid), and the ecotoxicity assessments in cyanobacteria appear to be based on the pro-drug only, may explain why cyanobacteria were relatively insensitive. In no cases were the chronic NOECs for invertebrates lower than the NOECs for cyanobacteria (Figure 4). The daphnid EC50 for the antifolate trimethoprim, however, was lower than the EC50 for cyanobacteria (8.21 and 91.68 mg/L, respectively. Figure S3). This was not the case for the NOECs for the same compound, indicating differences in the shape of the dose-response curve. Importantly, in this case cyanobacteria would still drive the $PNEC_{SW}$.

5.2 PNEC comparisons

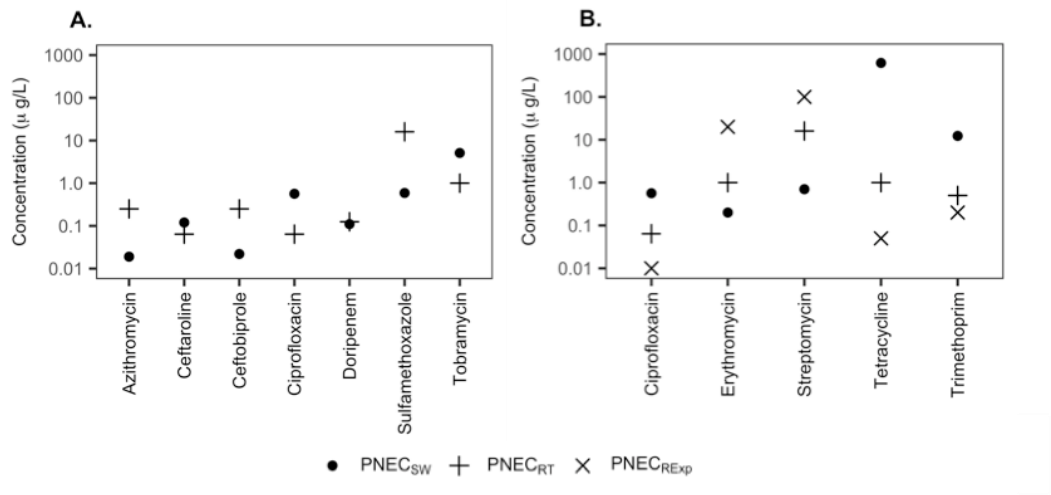

Figure 5. Comparisons of predicted no effect concentrations (PNEC) for antimicrobial resistance and ecotoxicity for aquatic taxa in surface water. A) Comparison of theoretically derived PNEC for resistance development ($PNEC_{R(T)}$) based on clinically relevant bacteria (Bengtsson-Palme and Larsson 2016) and PNEC for ecotoxicity in surface water ($PNEC_{SW}$). (B) Comparison of $PNEC_{R(T)}$, $PNEC_{R}$ based on experimentally derived minimum selective concentrations ($PNEC_{R(EXP)}$) and $PNEC_{SW}$. In A) data are presented for antibiotics only where a full data set including cyanobacteria, invertebrate and fish tests were available and calculated from no observed effect concentrations as described in (EMA 2006). $PNEC_{SW}$ in B) are calculated from cyanobacteria NOECs regardless of a complete ecotoxicity data set where a $PNEC_{R(EXP)}$ was available. $PNEC_{R(EXP)}$ is a less than (<) value in erythromycin and trimethoprim. $PNEC_{R(EXP)}$ based on strain specific MSC in ciprofloxacin, erythromycin, streptomycin and trimethoprim. $PNEC_{R(EXP)}$ based on community based MSC in tetracycline. EC50 for cyanobacteria was used because NOEC were not available for $PNEC_{SW}$ in streptomycin and tetracycline therefore NOEC may be up to an order of magnitude lower.

For the limited number of antibiotics where a definitive $PNEC_{SW}$ could be calculated (n=7) an analysis of the relationship between traditional ERA PNECs and those for AMR was conducted. Within this meta-analysis the theoretically determined PNEC for resistance development $PNEC_{R(T)}$) obtained from Bengtsson-Palme and Larsson (2016) for the different antibiotics was not always protective of (lower than) the $PNEC_{SW}$ (Figure 5A). The $PNEC_{R(T)}$ was lower than $PNEC_{SW}$ for ceftaroline, ciprofloxacin and tobramycin. However, the $PNEC_{SW}$ was approximately ten-fold lower than $PNEC_{R(T)}$ for ceftobiprole, sulfamethoxazole and azithromycin.

576

577 Where experimentally derived MSCs existed, the $PNEC_{R(Exp)}$ was lower than
578 $PNEC_{R(T)}$ for three out of five antibiotics with available data (Figure 5B).
579 However, $PNEC_{R(T)}$ overestimated the risk of resistance development for
580 streptomycin by an order of magnitude. $PNEC_{R(T)}$ and $PNEC_{R(Exp)}$ were similar for
581 trimethoprim (Figure 5B; trimethoprim $PNEC_{R(Exp)}$ was <0.2 μg/L). The $PNEC_{SW}$
582 for erythromycin and streptomycin were lower than their $PNEC_{R(T)}$ and
583 $PNEC_{R(Exp)}$ (Figure 5B). The $PNEC_{R(Exp)}$ for erythromycin however, did not have a
584 definitive value, (i.e. <0.2mg/L) and as such we assign caution to this
585 comparison.

5.3 Establishing 5th percentiles

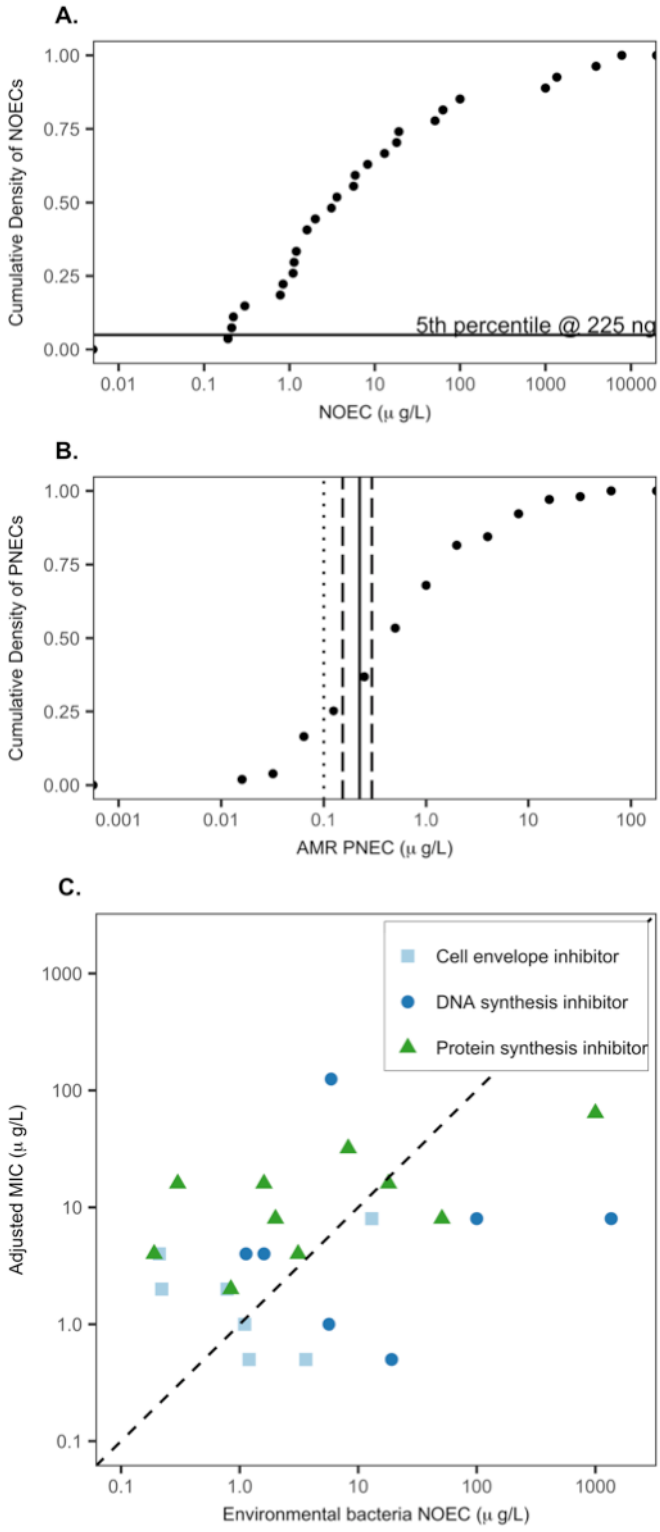

Figure 6. A) Cumulative density plot of the NOECs for environmental bacteria for 27 antibiotics, showing the 5th percentile. B) Cumulative density plot of PNECs for AMR for 103 antibiotics, as calculated by Bengtsson-Palme and Larsson (2016). The vertical solid line represents the 5th percentile of the bacteria NOECs, dashed lines represent the standard error and dotted line indicates the proposed discharge limit. Note each point can represent up to 17 antibiotics. C)

Comparison of NOECs for environmental bacteria and clinically relevant bacteria minimum inhibitory concentrations.

We determined the 5th percentile for growth inhibition data for cyanobacteria and environmental bacteria and MICs for CRB (See table S4). The rationale for this was to establish an environmental protection goal for antibiotic production discharges that would be protective of bacterial NOECs with 95% confidence. The 5th percentiles ranged from 225 to 2028 ng/L, depending on the bacteria and endpoints used. The lowest NOECs for environmentally relevant bacteria (cyanobacteria, *P. putida* and *V. fischeri*) gave the lowest value (225 ± 71 ng/L, Figure 6A).

6 Discussion

In our evaluation of the current regulatory ERA guidance we show that of the taxa tested, as expected based on the mechanisms of action, prokaryotes were most sensitive to antibiotics. However, we also show that reliance on one species of (cyano)bacteria to set protection levels (e.g. PNECs), as operates currently, is unlikely to be protective of environmental and human health (through AMR). Individually, neither traditional aquatic PNECs nor the AMR based PNECs protect fully against the effects of antibiotics. We thus recommend the inclusion of both clinically important bacteria and a wider range of species of environmentally relevant bacteria to improve the prospective regulatory framework for human and ERA. This approach will help also in defining more appropriate safe discharge concentrations for antibiotic production, and help to exclude unnecessary ERA testing on metazoan species.

6.1 Species relative sensitivity: the need for more bacteria

During their development, the efficacy and safety of new antibiotics are assessed in preclinical and clinical studies before market approval. It is therefore unlikely that toxic effects will occur in an aquatic vertebrate (such as fish) at water concentrations lower than those affecting prokaryotic species (target or non-target). As expected, in our analyses, those species evolutionarily more distant to pathogenic bacteria were generally less sensitive to antibiotics compared with

624 clinically relevant and environmental bacteria. Our results also indicate that
625 neither cyanobacteria, CRB nor other environmental bacteria (*V. fischeri* and *P.*
626 *putida*) provide a single organism/test that is fully protective of the diversity of
627 bacteria in the environment. Thus, a $PNEC_{SW}$ determined according to the
628 current ERA guidance (EMA 2006; US Food and Drug Administration 1998) will
629 not always be protective of the environment.
630
631 Sensitivity to any one antibiotic differed by up to five orders of magnitude across
632 different species of cyanobacteria. Patterns of sensitivity for the different genera
633 were observed across the different antibiotic mechanisms of actions, but no one
634 species was consistently the most sensitive. Cyanobacteria are one of the most
635 diverse phyla on the planet (Shih et al. 2013; Whitton 2012) and this large range
636 in sensitivity to antibiotics might therefore be expected. In ERA *A. flos-aquae* is
637 the most regularly used of the two OECD test guideline recommended
638 cyanobacterial species (the other being *S. leopoliensis;* (OECD 2011)) but *A. flos-*
639 *aquae* was the most sensitive cyanobacteria for only one of the 13 antibiotics for
640 which data were available for multiple genera and species. In the cases of
641 ampicillin, erythromycin, norfloxacin, oxytetracycline, sulfdiazine and
642 trimethoprim (35% of antibiotics with multiple cyanobacterial EC50s) the
643 difference in sensitivity between *A. flos-aquae* and the most sensitive taxon was
644 greater than the assessment factor (x10) used to generate a PNEC for the risk
645 assessment. For ampicillin, reliance on *A. flos-aquae* could underestimate the
646 $PNEC_{SW}$ by more than three orders of magnitude. This questions the current over
647 reliance on a single cyanobacteria test species within ERA frameworks and we
648 propose at least three cyanobacteria genera should be included within these risk
649 assessment frameworks. The case above for ampicillin highlights a further
650 important issue relating to the relevance of high sensitivity for some
651 cyanobacteria. Ampicillin is not persistent in the environment and undergoes
652 partial degradation by bacteria; indeed, primary degradation is the resistance
653 mechanism. If degradation were factored in, from an ecotoxicological point of
654 view, exposure and environmental effects would be low, although community
655 structure changes could impact resilience. Furthermore, since the resistance
656 mechanism partially degrades the antibiotic resulting in a lower concentration of

ampicillin in the environment care needs to be taken not to assume a low measured concentration of ampicillin necessarily equates with an absence of selection for AMR development and human health risk.

The cyanobacteria adopted for toxicity testing has been based largely on experimental convenience (e.g. the ability to grow them and measure cell density in the laboratory) with little knowledge on how representative they are of other cyanobacteria. No consideration has been given to how they grow and function in non-pelagic habitats, e.g. biofilms. From our analyses, *M. aeruginosa* would potentially provide a relatively high sensitivity to most antibiotics. This species however, has a slower growth rate and the current test with this species may therefore have to be extended to make the test comparable in terms of the growth and replication dynamics with that for *A. flos-aquae* and *S. leopoliensis*. We highlight that the requirement for optimised conditions for culturing a species and variation in life history components across species (e.g. growth rates and lag time) create further challenges for interspecies substance effects analyses. For example, exposure time can have a direct impact on the perceived sensitivity. In this meta-analysis we have used data that are based on regulatory approved guidelines in which exposure time and exposure conditions have been optimized for the different organisms to ensure that growth in the controls do not reach the plateau phase, thus maximizing the ability to detect for any effects against treatment groups. Longer exposure periods could potentially result in lower effective exposure concentrations, as we demonstrate for the EC50 in *V. fischeri* (for a 24 hour exposure compared with shorter test periods) and as has been shown for the ASRIT (Kümmerer et al. 2004)). Extending exposure periods in growth tests however needs to ensure that this does not compromise the ability to distinguish for effects i.e. additional time does not result in the controls being limited in their growth dynamics by the available resources and thus affect the comparison with the treated groups. It needs to be recognized, however, that differences between test conditions optimized for different species (e.g. chemical constituents of the culture media, pH, temperature, light intensity and test length, to name just a few) could all impact the fate and behavior of the antibiotic and its bioavailability, distribution, metabolism and excretion in test organisms,

690 which in turn may influence the perceived relative sensitivity. Distinction needs
691 to be made on whether the exposure adopted is optimized for assessment of
692 effects relative to controls (as is the case in the OECD 201 test guideline for green
693 algae and cyanobacteria) or focused more on environmental relevance (for
694 example in the ASRIT analyzing for impacts within hydraulic residence time in
695 sewerage treatment works). Species sensitivity analyses and /or functional
696 impacts are arguably better addressed under context specific conditions that
697 consider the microbial community structure(s) and physicochemical conditions
698 that occur in those natural systems.
699
700 Available study information was not sufficiently comprehensive to allow for
701 consideration of these variables within our meta-analysis and we were thus
702 restricted to endpoint data (EC_{50} and NOEC) that we derived from reliable
703 studies. Further investigation is warranted into the physiological basis for the
704 differences in sensitivity to antibiotics to help identify species, or groups of
705 species, that best represent the phylum for their protection and the critical
706 ecosystem services (e.g. primary productivity and food source) they provide.
707
708 *V. fischeri* and *Pseudomonads* were more sensitive than cyanobacteria to some
709 antibiotics and may potentially provide valuable additional species for inclusion
710 within the ERA. Furthermore, they already have internationally recognised test
711 guidelines (ISO 1995; 2007). *V. fischeri*, is a marine bacterium that would not
712 normally be considered in ERA for freshwaters, but is sometimes used in whole
713 effluent assessments (ECETOC 2004). It is, nevertheless, a prokaryotic species
714 and antibiotics and antibiotic resistant bacteria have been detected in estuaries
715 and marine environments emanating from sewerage treatment plant discharges
716 and manufacturing effluents (Schaefer et al. 2009; Webster et al. 2004; Zheng et
717 al. 2011; Zou et al. 2011). The compiled data show that *V. fischeri* was more
718 sensitive than cyanobacteria for six antibiotics, and for half of these by nearly an
719 order of magnitude (flumequine, lomefloxacin and oxolinic acid). The inclusion
720 of this test could therefore be of value to ERA if performed with an exposure time
721 of 24 hours (results based on exposure lengths of less than 24 hours showed
722 significantly less sensitivity). *Pseudomonads* have been shown to be less

723 sensitive than the other soil bacteria to tetracycline, chlortetracycline, and
724 oxytetracycline and in some instances by over an order of magnitude (Halling-
725 Sørensen et al. 2002). The low sensitivity observed in *Pseudomonas* species has
726 been attributed to their apparent high natural resistance to some antibiotics
727 (Halling-Sørensen et al. 2002; Kittinger et al. 2016). Thus, our findings suggest
728 that additional testing with *P. putida* could be of value to the ERA, but it may still
729 not be protective of other soil bacteria. Any consideration to incorporate the test
730 with *P. putida* in antibiotic ERA would need to first characterise the strain in
731 terms of its chromosomal and plasmid resistance to help prevent biasing any
732 function or growth based assessment (Brandt et al. 2015).
733
734 The ASRIT (OECD 2010) was several orders of magnitude less sensitive to
735 antibiotics than cyanobacteria and other bacterial species, confirming reports
736 that this test is largely insensitive to antibiotics (Kümmerer et al. 2004). As such,
737 the ASRIT would not influence the outcome of the ERA. This lack of sensitivity
738 may be due to several factors, including the short exposure time (3 hour) of the
739 test (Kümmerer et al. 2004), the lack of antibiotic bioavailability due to
740 adsorption to the sludge solids (e.g. Golet et al. 2002) or that the microbial
741 community in the activated sludge has an innate resistance having been exposed
742 previously to the antibiotic (Davies 2012). It was not possible to assess the effect
743 of extending the ASRIT test duration due to a lack of available data and because
744 most ASRIT results are reported as censored data of >100 mg/L. Furthermore,
745 the endpoint of respiration, may not be suitable for all mechanisms of actions
746 (Brandt et al. 2015) and it does not equate with changes in bacterial diversity or
747 community structure. We thus support the need to replace and/or complement
748 the ASRIT with other assays (Brandt et al. 2015), which are relevant for all
749 pharmaceuticals.
750
751 In order to build greater confidence in the ERA for antibiotics we sought to gain a
752 better understanding on the differences observed in sensitivity between the
753 species and to establish both how often and for which antibiotic classes these
754 differences exceed the assessment factor of 10. Overall, across all the antibiotics
755 assessed, cyanobacteria and CRB were equally sensitive to antibiotics (figure 1).

Thus, neither CRB nor cyanobacteria were consistently more sensitive than the other. In this meta-analysis, the inclusion of CRB in ERA would drive the PNEC in 40% of cases further supporting a more holistic 'one health' approach that uses clinical and environmental data. There were, however, substantial differences in sensitivity to antifolates observed between the cyanobacterial species and CRB. The folate synthesis pathway that antifolates inhibit is present in cyanobacteria and so the reason for the apparent lack of sensitivity in some cyanobacteria is unknown. However, de Crécy-Lagard et al. (2007) reported that cyanobacteria possess a protein that may act as a folate transporter allowing the bypassing of some of the folate synthesis pathway. Our analysis suggests therefore that cyanobacteria may not always be a suitable representative for bacteria for full protection against antifolate antibiotics.

Macrophytes appear especially sensitive to antifolates and quinolones. The folate synthesis pathway in bacteria, algae and plants is fundamentally the same (Basset et al. 2005) and they are, therefore, all potentially susceptible to antifolates. Indeed, sulfamethoxazole has been reported to act as a competitive agonist to *p*-aminobenzoic acid in both *Lemna gibba* (Brain et al. 2008b) and *Arabidopsis thaliana* (Zhang et al. 2012). Macrophytes were also more sensitive than cyanobacteria to five quinolones. Quinolones cause toxicity by forming complexes with DNA gyrase or topoisomerase IV resulting in the inhibition of DNA replication and transcription (Aldred et al. 2014). Chloroplasts are descended from cyanobacteria (Falcon et al. 2010) and some plants and red algae have been shown to contain DNA gyrases in their plastids (including chloroplasts) and mitochondria (Moriyama and Sato 2014; Wall et al. 2004). Quinolone antibiotics are reported to have anti-chloroplastic activity (Brain et al. 2008a; Brain et al. 2004; Ebert et al. 2011) which can affect photosynthesis in plants (Brain et al. 2008a). Indeed, organellar DNA gyrase has been shown to be the primary target of ciprofloxacin in *Arabidopsis thaliana* (Evans-Roberts et al. 2016). Thus, our findings indicate that for some antibiotics in these classes, macrophytes could potentially drive the protection goal. Consequently, these species should be considered for inclusion within risk assessment frameworks for antibiotics.

The metazoan taxa were never found to be the most sensitive compared with all bacterial taxa. This questions the necessity of resource intensive metazoan testing of antibiotics, as required by European Medicines Agency and Food and Drugs Administration guidance (EMA 2006). Inclusion of appropriate (and additional) bacterial testing in the ERA for antibiotics would potentially allow for the exclusion of some unnecessary testing on metazoan species, acknowledging the principles of the 3R's to replace, reduce and refine studies that use 'protected' animals, such as fish (Hutchinson et al. 2016; Scholz et al. 2013).

We performed this meta-analysis based on data that was deemed most reliable according to the CRED system (Moermond et al. 2016). The conclusions however, are still drawn upon data that were conducted in different labs, with different procedures and of varying quantity (in terms of test performance and meta-data) and quality of reporting. We strongly emphasise the need to collect and report suitable control data, chemical analysis and meta-data in order to assist in reliable comparisons of studies.

An analysis of appropriate additional bacterial species for inclusion in the ERA needs to consider potential differences in sensitivity due to pharmacokinetic considerations including bioavailability, charge, uptake, elimination, metabolism, degradation rates or binding affinities, or a combination of them. Differences in bacterial morphologies and innate resistance may also account for some of the differences in sensitivity between species. Some bacteria have several different growth forms depending on the environmental conditions. As an example, increased temperature and light intensity causes aggregation of *Synechococcus elongates* cells (Koblížek et al. 2000) and this aggregation may have an impact on the sensitivity of the cells to antibiotic exposure. Several studies have demonstrated that cells in biofilms are less sensitive/more protected from chemical exposure (Balcázar et al. 2015). A better understanding of how physiological and morphological differences in cells and community structure affect the toxicity of chemicals to bacteria is required to fully understand the risk posed by antibiotics in the environment.

Bacteria are fundamental to many vital ecosystem services, but little is understood regarding species loss and functional redundancy and thus, the resilience of ecosystem function. Some investigators, however, have begun to address this. For example, Lundström et al. (2016) found no change in the overall taxonomic diversity when biofilms were exposed to tetracycline, however, the community composition was altered and the functional diversity, as measured by utilization of carbon sources, decreased with increasing tetracycline concentrations. Ciprofloxacin exposure altered the bacterial community structure in marine sediments at 0.2 mg/L), resulting in a decrease in the community ability to degrade pyrene (Näslund et al. 2008). It was also found to increase overall biomass in salt marsh microbial communities, favouring gram negative and sulfate-reducing bacteria (Cordova-Kreylos and Scow 2007). Several studies have shown that bacterial diversity has a positive relationship with ecosystem function (Bell et al. 2005; Langenheder et al. 2010). Delgado-Baquerizo et al. (2016) demonstrated that loss of diversity in aquatic bacterial communities caused a decrease in both broad (microbial respiration) and specialized (toxin degradation; of mycrocystin-LR and triclosan degradation) endpoints and the communities showed little or no functional redundancy. These studies indicate that a small drop in bacterial diversity may potentially impact negatively on the ecosystem services they provide.

From this, we conclude that the ERA framework for antibiotics needs to be based upon a suitable range of bacteria. This should include CRB and capture a wider range of ecologically important functional groups. Previous investigators have identified standard studies that may fulfill some of these data gaps e.g. nitrifying bacteria, methanogens and sulfate-reducing bacteria (Brandt et al. 2015) although more research is required to identify if these tests will be protective of all functional bacterial groups or if further standard tests will need to be developed. The effect of antibiotics on these functional groups is currently outside risk assessment frameworks and environmental and non-therapeutic human impacts are considered in isolation. Furthermore, a measure of the change in community structure would add value, especially looking at diversity

in terms of clinical and environmental relevance, and understanding to changes in functional endpoints in bacterial multispecies/community tests to determine whether ecological resilience is being compromised.

6.2 PNECs for AMR verses traditional ecotoxicological effects

AMR is a serious risk to human health globally and currently sits outside the ERA regulations. Both theoretical methodologies and empirical data available for assessing AMR selection and transfer in the environment are limited. Consequentially, evidence is lacking to assess the best approach for the risk of AMR development, how resistance in the environment may lead to enrichment of resistance in human pathogens and how the risk posed by antibiotics by AMR development compares to their effects upon ecosystem function and services. Previous investigators have explored resistance selection using a variety of approaches, for example, comparing predicted environmental concentrations with MICs (Kümmerer and Henninger 2003), using MICs to calculate potentially affected fractions of communities (Singer et al. 2011) and using growth and competition experiments to demonstrate resistance selection (Negri et al. 2000) and calculate MSCs (Gullberg et al. 2011). The theoretical approach proposed by Bengtsson-Palme and Larsson (2016) is a recent contribution and provides a good basis for this discussion, using MIC data to assess reduction in antibiotic efficacy due to erosion by resistance. However, it is important to note that this approach assumes growth can be used to predict resistance and is not verified through direct testing of resistance markers and as such any conclusions drawn from this analysis must therefore be considered with this in mind.

Our findings suggest that the $PNEC_{RT)}$ defined by Bengtsson-Palme and Larsson (2016) is not always lower than the $PNEC_{SW}$; for 7 antibiotics $PNEC_{SW}$ was lower in four cases (figure 5). This may be due to either the $PNEC_{R(T)}$ underestimating the risk or cyanobacteria being more sensitive to some antibiotics compared with the CRB. Experimentally determined MSCs were derived largely from laboratory strain competition experiments (four of the five cases; Figure 5B), where strains that differ in only the presence/absence of the resistance genes under investigation are compared (Gullberg et al. 2014; Gullberg et al. 2011).

887 These strain competition experiments have limitations in scaling up to more
888 complex microbial communities (Bengtsson-Palme et al. 2014). There are very
889 few cases where analyses have been conducted for more complex communities
890 but it is hypothesised that the combined effects of changes in community
891 structure (due to loss of the most sensitive species), protective morphological
892 forms (e.g. bacteria maybe less susceptible in biofilms compared to those within
893 the water column (Balcázar et al. 2015)), difficulty in defining the 'true' antibiotic
894 exposure concentration, and alternative selection pressures (e.g. nutrient
895 limitation, predation and other chemical/physical stressors) may negate the
896 fitness benefit of the resistance (Bengtsson-Palme and Larsson 2016; Brosche
897 and Backhaus 2010; Day et al. 2015; Gullberg et al. 2014; Lundström et al. 2016;
898 Quinlan et al. 2011). Most studies that have considered effects of antibiotics on
899 complex communities have been taxon independent, assessing AMR gene copy
900 number relative to 16SrRNA, rather than providing species specific information.
901 Investigations into AMR following tetracycline exposure, however, have found
902 that resistance was increased in periphyton at the lowest test concentration of
903 0.5 µg/L (Quinlan et al. 2011), horizontal gene transfer (HGT) was promoted at
904 10 µg/L (Jutkina et al. 2016) and resistant bacteria and resistance genes was
905 increased in biofilms at concentrations below 1 µg/L (Lundström *et al* , 2016).
906 Assuming an assessment factor of 10, from this data a $PNEC_{R(Exp)}$ would be 0.05
907 µg/L, which is 20 times lower than $PNEC_{R(T)}$ of 1 µg/L (Bengtsson-Palme and
908 Larsson 2016). There is no NOEC data for tetracycline in cyanobacteria, but in
909 *Microcystis aeruginosa* a EC50 is reported at 90 µg/L (Halling-Sørensen, 2000)
910 and in *Anabaena* sp an EC10 of 2.5 mg/L (González-Pleiter et al. 2013),
911 suggesting that resistance for tetracycline may occur at concentrations nearly
912 100-fold lower than effects on growth inhibition in cyanobacteria. This again
913 emphasizes the need for a more holistic approach to the setting of protection
914 goals for antibiotics and the development of validated assays to assess MSCs in
915 complex and simple systems, as well as generating toxicity data for
916 cyanobacteria and other environmental and/or clinical bacteria.
917
918 It should be recognized that although studies that are used to guide regulatory
919 decision-making require standardized test methodologies to help ensure reliable

and repeatable results, the link between these single species studies and those operating in the complex systems in the field is largely unknown and, as mentioned previously, the link to ecosystem services is not made. The application of mesocosm studies that enable community response and effects upon ecosystem functions to be assessed have good utility here to help provide insights into the development of AMR in environmentally realistic scenarios (Knapp et al., 2008; Knapp et al., 2010; Quinlan et al., 2011). In addition to living in complex communities in the environment, it is important to note that organisms are also likely to be exposed to antibiotic mixtures and the relationship between single exposure laboratory testing and mixtures toxicity is unknown and requires further research (Backhaus et al. 2000; Brosche and Backhaus 2010; González-Pleiter et al. 2013; Liu et al. 2014).

In the context of current regulatory guidance, MSCs derived from experimental data, albeit they are limited, in some cases supported the theoretically derived $PNEC_{R(T)}$. There were cases also where $PNEC_{R(T)}$ was not necessarily appropriate (optimal) for risk assessment for AMR. Nevertheless, until there is an internationally accepted method for the experimental determination of $PNEC_R$ - which may require further knowledge on resistance mechanisms, model variability and the application to mixed communities that vary over time and space - the theoretical approach advocated by Bengtsson-Palme and Larsson (2016), based on MIC data in the European Committee on Antimicrobial Susceptibility Testing database, provides a valuable alternative as part of a broader evidence-based approach to ERA. Moreover, it provides an efficient and cost effective method to address concerns and prioritise legacy antibiotics that have already been registered and are present in the environment. It should be noted, however, that there are clear limitations to this approach (as identified by the paper's authors). These include the test conditions for determining the MIC in CRB, that are largely environmentally irrelevant, the assumptions that growth inhibition can be used to predict selection for resistance. There is also an assumption that an assessment factor of 10 will provide a suitable safety margin to account for selection below the MIC and conversely that adjusting the MIC down to account for species numbers and then applying a further assessment

factor of 10 isn't overprotective. Finally, MIC-derived protection goals will change over time, as MICs are determined for more species with variable sensitivity and as a consequence periodic updates will be required.

Our analysis suggests that the susceptibility of species in European Committee on Antimicrobial Susceptibility Testing is not always protective of environmental bacteria, such as cyanobacteria and therefore a $PNEC_{R(T)}$ using CRB MIC data as a surrogate for resistance may not be protective of the risk of AMR development in environmental bacteria. Furthermore, we show that a $PNEC_{R(T)}$ may not be protective of ecosystem function traditionally determined using the growth inhibition test with cyanobacteria. From this we conclude that despite evidence that resistance will occur at lower concentrations than the effects on population density (Gullberg et al. 2011; Hughes and Andersson 2012), both a $PNEC_R$ and a $PNEC_{SW}$ are needed to establish safe concentrations for the protection of ecosystem function and against the development of resistance.

It is noteworthy that from an environmental health perspective (rather than human health), AMR can provide an ecosystem service or benefit. For example, bacteria expressing beta-lactamase enzyme activity degrade and reduce the environmental burden of beta-lactam antibiotics and this in turn could contribute positively in sewerage treatment plants where high antibiotic concentration might otherwise compromise functional efficiency.

6.3 Production discharge limits

In addressing the impact of antibiotic pollution on ecosystem function, AMR development and human health, safe discharge limits for antibiotic production facilities need to be established (Agerstrand et al. 2015; Larsson 2014; Pruden et al. 2013). However, there are few data available in the public domain to support the development of such limits and this is especially so for experimental data on AMR development. Most data that are available are based on growth inhibition tests and we have therefore identified the lowest NOEC values for 27 antibiotics representing sensitive phyla (cyanobacteria, *V. fischeri* and *P. putida*) and using these data we estimate the 5^{th} percentile to be 225 ± 71 ng/L. Thus, a

conservative limit of 154 ng/L would account for uncertainty. Provided that these 27 antibiotics are representative of all antibiotics, the cyanobacterial NOECs are, with 95% confidence, likely to be higher than 154 ng/L.

The lowest MSC reported in the literature is 100 ng/L with many others between 10-1000 times higher (Brosche and Backhaus 2010; Gullberg et al. 2014; Gullberg et al. 2011; Lundström et al. 2016). Setting a threshold limit of 100 ng/L for antibiotic discharges would, therefore, appear to be protective of environmental bacterial populations (with 95% confidence) and match the lowest empirical evidence of AMR development. However, it would not be protective for 16% of the theoretical $PNEC_{R(T)}$s, described by Bengtsson-Palme and Larsson (2016) (Figure 6B) highlighting that safe discharge limits may need to be lower than this for some antibiotics in order to consider the potential to select for resistance in clinical and environmental isolates. It should be noted, however, that the $PNEC_{R(T)}$ incorporates a correction factor that adjusts the MIC according to the number of species it is based upon and a further assessment factor of 10 to account for AMR. In turn, the corrections could cause the $PNEC_{R(T)}$ to be over protective (as shown for some antibiotics in Figure 5B).

A single, protective threshold limit that could be applied as an interim measure in the absence of other reliable empirical clinical and or environmental data (and standardised methodologies for AMR), which is based on empirical data would be of great value. Based on the antibiotic compounds for which we were able to obtain NOECs from environmentally relevant bacteria and from the available MSCs in the literature, we suggest a production discharge limit of 100 ng/L for each antibiotic, applied in the mixing zone downstream of the point source discharge for protection of ecosystem function and the risk of AMR development. The use of a single protection goal rather than a range, for production facilities offers pragmatic benefits to industry and suppliers. Compliance with a single protection value provides simplicity and ease of implementation compared with the 111 values advocated for the different antibiotics suggested by Bengtsson-Palme and Larsson (2016), of which some would not be protective of the environment or the MSC. Consideration is required for how this limit would

apply in the case of antibiotic mixtures, although this falls out of scope of this meta-analysis.

This approach could also help prevent the use of conflicting values for a single antibiotic. However, it is important to ensure that this value proves to be protective. So where other data are available (e.g. empirical or $PNEC_{R(T)}$) that suggest a lower limit is required to be protective, the 100 ng/L should be adjusted accordingly to provide the required protection. Equally, a higher limit may be applicable where there are substantive data to support its increase. We advocate this as an interim measure only until more data are obtained to support the risk analysis for antibiotics. Furthermore, as methodologies for the assessment of AMR are developed these values should also be incorporated and protection goals updated.

7 Concluding remarks and considerations for ERA

Our analysis shows that frameworks for ERA and human health protection (through protection for the risk of AMR) for antibiotics need to consider the impact of antibiotics on relevant vulnerable species and the essential ecosystem services they provide. The current framework for ERA based on just one cyanobacterial species is, in many cases, inadequate and it does not address risk to critical ecosystem services. There is also an urgent need to better establish the effects of antibiotics on bacterial diversity, community structure, ecosystem function and resilience in order to better understand the effects of antibiotics in the environment.

We emphasise that the presence of antibiotics in the environment does not necessarily lead to the development of AMR in bacterial communities and studies are required that better establish the toxic effects of antibiotics, AMR and the relationship between them in environmentally relevant contexts. In the environment other selection pressures (e.g. nutrient availability and predation) may be more significant than that posed by exposure to low levels of antibiotics. As a consequence AMR may not be observed at the same concentrations as in the

laboratory studies. However, it is also the case that the fitness cost of carrying some resistance genes may be very low or even neutral and therefore the genes coding for resistance could remain in the bacterial communities after only a short exposure. Understanding these complexities in AMR development in the environment is crucial for establishing interrelationships with human pathogens and in turn managing and mitigating the risk of antibiotics in the environment for the protection of human health.

From our analyses on relative species sensitivity we highlight the following as key considerations for the use, and development of human and ERA frameworks for antibiotics.

1. The need for inclusion of a larger selection of bacterial species for testing to account for the variability in sensitivity between species and for greater confidence in the protection of bacterial communities and the ecosystem services they provide.
    a. Brandt et al. (2015) have identified a number of suitable established standard tests for other bacteria (including *P. putida*) and for ecosystem services (e.g. nitrification and carbon transformation) and these should be considered as additional tests in the ERA of antibiotics.
    b. We show that pre-clinical MIC data of CRB could be used to increase the diversity of bacterial species represented in ERA at little cost. The use of pre-clinical and clinical data is often advocated to identify environmental risk (Boxall et al. 2012) but the realisation of this is limited with 'bridging' studies and methods still being developed.
    c. We reaffirm that the only required community test, the ASRIT, is not sensitive to antibiotics and thus its suitability for determining the effect of antibiotics to environmental bacteria and sewerage treatment plant microorganism communities is questionable. Consideration for its replacement by tests to assess the effects on bacterial community function or impacts on population growth are warranted.

2. Testing of antibiotics on metazoans may not be required.
    a. Metazoans were generally 2 to 4 orders of magnitude less sensitive to antibiotics than cyanobacteria. Further investigation is required to assess and confirm these results on a wider series of empirical *in vivo* exposures, however this meta-analysis provides a starting point for this discussion and the possible reduction in the use of metazoans in antibiotic testing.
3. Our meta-analysis highlights that the relative high sensitivity of microalgae and macrophytes to some antifolate and quinolone antibiotics (compared with cyanobacteria) supporting their inclusion in risk assessment frameworks for these compound classes. Further research into the relative sensitivity of macrophytes and microalgae to these classes of antibiotics is warranted.
4. Test systems to determine PNEC or MSC for AMR development are urgently required for clinical and environmental species. Our analysis, suggests that the CRB in the European Committee on Antimicrobial Susceptibility Testing database are not always representative of the diversity of sensitive bacteria in nature. This illustrates that ERA needs to incorporate both $PNEC_{SW}$ and $PNEC_{R}$. There is a need to develop a standardised method to experimentally determine an MSC in environmental and clinical bacteria, exemplified by three out of five experimental values being lower than the theoretical value.
5. A discharge limit of 100 ng/L maybe a protective and pragmatic approach to address environmental concerns around antibiotic production in the absence of sufficient reliable clinical and environmental data, whilst urgently needed methodologies and empirical data are obtained to draw firmer conclusions. Where data exists that suggest a higher or lower concentration is required to be protective that value should be used instead.

8 Acknowledgments

We thank James Cresswell for his assistance with the statistical modelling and MSD for providing additional antibiotic data. The trade organisation for the

research-based pharmaceutical industry in Sweden (LIF) provided a list of all active pharmaceutical ingredients with environmental data in www.fass.se as of 01/12/2016.

9 References

Agerstrand, M.; Berg, C.; Bjorlenius, B.; Breitholtz, M.; Brunstrom, B.; Fick, J.; Gunnarsson, L.; Larsson, D.G.J.; Sumpter, J.P.; Tysklind, M.; Ruden, C. Improving Environmental Risk Assessment of Human Pharmaceuticals. Environmental Science & Technology 2015;49:5336-5345

Aldred, K.J.; Kerns, R.J.; Osheroff, N. Mechanism of Quinolone Action and Resistance. Biochemistry 2014;53:1565-1574

Ando, T.; Nagase, H.; Eguchi, K.; Hirooka, T.; Nakamura, T.; Miyamoto, K.; Hirata, K. A novel method using cyanobacteria for ecotoxicity test of veterinary antimicrobial agents. Environmental Toxicology and Chemistry 2007;26:601-606

Ashbolt, N.J.; Amézquita, A.; Backhaus, T.; Borriello, P.; Brandt, K.K.; Collignon, P.; Coors, A.; Finley, R.; Gaze, W.H.; Heberer, T. Human health risk assessment (HHRA) for environmental development and transfer of antibiotic resistance. Environmental Health Perspectives (Online) 2013;121:993

Backhaus, T.; Scholze, M.; Grimme, L. The single substance and mixture toxicity of quinolones to the bioluminescent bacterium Vibrio fischeri. Aquatic Toxicology 2000;49:49-61

Balcázar, J.L.; Subirats, J.; Borrego, C.M. The role of biofilms as environmental reservoirs of antibiotic resistance. Frontiers in Microbiology 2015;6:1216

Basset, G.J.; Quinlivan, E.P.; Gregory, J.F.; Hanson, A.D. Folate synthesis and metabolism in plants and prospects for biofortification. Crop Science 2005;45:449-453

Bates, D.; Maechler, M.; Bolker, B.; Walker, S. Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software 2015;67:1 - 48

Batt, A.L.; Kim, S.; Aga, D.S. Comparison of the occurrence of antibiotics in four full-scale wastewater treatment plants with varying designs and operations. Chemosphere 2007;68:428-435

Bell, T.; Newman, J.A.; Silverman, B.W.; Turner, S.L.; Lilley, A.K. The contribution of species richness and composition to bacterial services. Nature 2005;436:1157-1160

Bengtsson-Palme, J.; Alm Rosenblad, M.; Molin, M.; Blomberg, A. Metagenomics reveals that detoxification systems are underrepresented in marine bacterial communities. BMC Genomics 2014;15:1-17

Bengtsson-Palme, J.; Larsson, D.G.J. Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. Environment International 2016;86:140-149

Boxall, A.; Rudd, M.A.; Brooks, B.W.; Caldwell, D.J.; Choi, K.; Hickmann, S.; Innes, E.; Ostapyk, K.; Staveley, J.P.; Verslycke, T. Pharmaceuticals and personal care products in the environment: what are the big questions? Environmental health perspectives 2012;

Brain, R.; Hanson, M.; Solomon, K.; Brooks, B. Aquatic Plants Exposed to Pharmaceuticals: Effects and Risks. in: Whitacre D., ed. Reviews of Environmental Contamination and Toxicology: Springer New York; 2008a

Brain, R.A.; Johnson, D.J.; Richards, S.M.; Sanderson, H.; Sibley, P.K.; Solomon, K.R. Effects of 25 pharmaceutical compounds to Lemna gibba using a seven-

1163 day static-renewal test. Environmental Toxicology and Chemistry
1164 2004;23:371-382
1165 Brain, R.A.; Ramirez, A.J.; Fulton, B.A.; Chambliss, C.K.; Brooks, B.W. Herbicidal
1166 effects of sulfamethoxazole in Lemna gibba: using p-aminobenzoic acid as
1167 a biomarker of effect. Environmental science & technology
1168 2008b;42:8965-8970
1169 Brandt, K.K.; Amézquita, A.; Backhaus, T.; Boxall, A.; Coors, A.; Heberer, T.;
1170 Lawrence, J.R.; Lazorchak, J.; Schönfeld, J.; Snape, J.R.; Zhu, Y.-G.; Topp, E.
1171 Ecotoxicological assessment of antibiotics: A call for improved
1172 consideration of microorganisms. Environment International
1173 2015;85:189-205
1174 Brosche, S.; Backhaus, T. Toxicity of five protein synthesis inhibiting antibiotics
1175 and their mixture to limnic bacterial communities. Aquatic Toxicology
1176 2010;99:457-465
1177 Brown, K.D.; Kulis, J.; Thomson, B.; Chapman, T.H.; Mawhinney, D.B. Occurrence
1178 of antibiotics in hospital, residential, and dairy effluent, municipal
1179 wastewater, and the Rio Grande in New Mexico. Science of The Total
1180 Environment 2006;366:772-783
1181 Cordova-Kreylos, A.L.; Scow, K.M. Effects of ciprofloxacin on salt marsh sediment
1182 microbial communities. The ISME journal 2007;1:585-595
1183 Davies, I.A. Effects of Antibiotics on Aquatic Microbes. Environment Department:
1184 University of York; 2012
1185 Day, T.; Huijben, S.; Read, A.F. Is selection relevant in the evolutionary emergence
1186 of drug resistance? Trends in Microbiology 2015;23:126-133
1187 de Crécy-Lagard, V.; El Yacoubi, B.; de la Garza, R.D.; Noiriel, A.; Hanson, A.D.
1188 Comparative genomics of bacterial and plant folate synthesis and salvage:
1189 predictions and validations. BMC Genomics 2007;8:245
1190 Delgado-Baquerizo, M.; Giaramida, L.; Reich, P.B.; Khachane, A.N.; Hamonts, K.;
1191 Edwards, C.; Lawton, L.A.; Singh, B.K. Lack of functional redundancy in the
1192 relationship between microbial diversity and ecosystem functioning.
1193 Journal of Ecology 2016;104:936-946
1194 Ebert, I.; Bachmann, J.; Kühnen, U.; Küster, A.; Kussatz, C.; Maletzki, D.; Schlüter,
1195 C. Toxicity of the fluoroquinolone antibiotics enrofloxacin and
1196 ciprofloxacin to photoautotrophic aquatic organisms. Environmental
1197 Toxicology and Chemistry 2011;30:2786-2792
1198 ECETOC. Whole Effluent Assessment. Brussels: European Centre for
1199 Ecotoxicology and Toxicology of Chemicals; 2004
1200 ECHA, M. Guidance on information requirements and chemical safety
1201 assessment. Chapter R 2008;10: Characterisation of dose [concentration]-
1202 response for environment
1203 EMA. GUIDELINE ON THE ENVIRONMENTAL RISK ASSESSMENT OF MEDICINAL
1204 PRODUCTS FOR HUMAN USE. CPMP/SWP/4447/00 Corr 2. 2006
1205 Evans-Roberts, K.M.; Mitchenall, L.A.; Wall, M.K.; Leroux, J.; Mylne, J.S.; Maxwell,
1206 A. DNA Gyrase is the Target for the Quinolone Drug Ciprofloxacin in
1207 Arabidopsis thaliana. Journal of Biological Chemistry 2015;
1208 Evans-Roberts, K.M.; Mitchenall, L.A.; Wall, M.K.; Leroux, J.; Mylne, J.S.; Maxwell,
1209 A. DNA Gyrase Is the Target for the Quinolone Drug Ciprofloxacin in
1210 Arabidopsis thaliana. The Journal of Biological Chemistry 2016;291:3136-
1211 3144

1212 Falcon, L.I.; Magallon, S.; Castillo, A. Dating the cyanobacterial ancestor of the
1213 chloroplast. ISME J 2010;4:777-783
1214 Finley, R.L.; Collignon, P.; Larsson, D.G.J.; McEwen, S.A.; Li, X.-Z.; Gaze, W.H.; Reid-
1215 Smith, R.; Timinouni, M.; Graham, D.W.; Topp, E. The Scourge of Antibiotic
1216 Resistance: The Important Role of the Environment. Clinical Infectious
1217 Diseases 2013;57:704-710
1218 Golet, E.M.; Alder, A.C.; Giger, W. Environmental Exposure and Risk Assessment
1219 of Fluoroquinolone Antibacterial Agents in Wastewater and River Water
1220 of the Glatt Valley Watershed, Switzerland. Environmental Science &
1221 Technology 2002;36:3645-3651
1222 González-Pleiter, M.; Gonzalo, S.; Rodea-Palomares, I.; Leganés, F.; Rosal, R.;
1223 Boltes, K.; Marco, E.; Fernández-Piñas, F. Toxicity of five antibiotics and
1224 their mixtures towards photosynthetic aquatic organisms: Implications
1225 for environmental risk assessment. Water Research 2013;47:2050-2064
1226 Gullberg, E.; Albrecht, L.M.; Karlsson, C.; Sandegren, L.; Andersson, D.I. Selection
1227 of a Multidrug Resistance Plasmid by Sublethal Levels of Antibiotics and
1228 Heavy Metals. mBio 2014;5
1229 Gullberg, E.; Cao, S.; Berg, O.G.; Ilbäck, C.; Sandegren, L.; Hughes, D.; Andersson,
1230 D.I. Selection of Resistant Bacteria at Very Low Antibiotic Concentrations.
1231 PLoS Pathog 2011;7:e1002158
1232 Halling-Sørensen, B.; Sengeløv, G.; Tjørnelund, J. Toxicity of tetracyclines and
1233 tetracycline degradation products to environmentally relevant bacteria,
1234 including selected tetracycline-resistant bacteria. Archives of
1235 Environmental Contamination and Toxicology 2002;42:263-271
1236 Harrell Jr, F. Hmisc: Harrell Miscellaneous. R package version 4.0-0.; 2016
1237 Hughes, D.; Andersson, D.I. Selection of resistance at lethal and non-lethal
1238 antibiotic concentrations. Current Opinion in Microbiology 2012;15:555-
1239 560
1240 Hutchinson, T.H.; Wheeler, J.R.; Gourmelon, A.; Burden, N. Promoting the 3Rs to
1241 enhance the OECD fish toxicity testing framework. Regulatory Toxicology
1242 and Pharmacology 2016;
1243 IFPMA. Industry Roadmap for Progress on Combating Antimicrobial Resistance
1244 http://wwwifpmaorg/wp-content/uploads/2016/09/Roadmap-for-
1245 Progress-on-AMR-FINALpdf 2016;
1246 ISO. Water quality -- Pseudomonas putida growth inhibition test (Pseudomonas
1247 cell multiplication inhibition test). ISO 10712 1995;
1248 ISO. Water quality -- Determination of the inhibitory effect of water samples on
1249 the light emission of Vibrio fischeri (Luminescent bacteria test) -- Part 1:
1250 Method using freshly prepared bacteria. ISO 11348-1 2007;
1251 Jutkina, J.; Rutgersson, C.; Flach, C.-F.; Joakim Larsson, D.G. An assay for
1252 determining minimal concentrations of antibiotics that drive horizontal
1253 transfer of resistance. Science of The Total Environment 2016;548–
1254 549:131-138
1255 Khan, S.; Beattie, T.K.; Knapp, C.W. The use of minimum selectable concentrations
1256 (MSCs) for determining the selection of antimicrobial resistant bacteria.
1257 Ecotoxicology 2017;26:283-292
1258 Kittinger, C.; Lipp, M.; Baumert, R.; Folli, B.; Koraimann, G.; Toplitsch, D.;
1259 Liebmann, A.; Grisold, A.J.; Farnleitner, A.H.; Kirschner, A.; Zarfel, G.

Antibiotic Resistance Patterns of Pseudomonas spp. Isolated from the River Danube. Frontiers in Microbiology 2016;7:586
Koblížek, M.; Komenda, J.; Masojídek, J.; Pechar, L. CELL AGGREGATION OF THE CYANOBACTERIUM SYNECHOCOCCUS ELONGATUS: ROLE OF THE ELECTRON TRANSPORT CHAIN. Journal of Phycology 2000;36:662-668
Kümmerer, K. Antibiotics in the aquatic environment – A review – Part I. Chemosphere 2009;75:417-434
Kümmerer, K.; Alexy, R.; Hüttig, J.; Schöll, A. Standardized tests fail to assess the effects of antibiotics on environmental bacteria. Water Research 2004;38:2111-2116
Kümmerer, K.; Henninger, A. Promoting resistance by the emission of antibiotics from hospitals and households into effluent. Clinical Microbiology and Infection 2003;9:1203-1214
Kuznetsova, A.; Brockhoff, P.; Christensen, R. lmerTest: Tests for random and fixed effects for linear mixed effect models (lmer objects of lme4 package). 2013
Langenheder, S.; Bulling, M.T.; Solan, M.; Prosser, J.I. Bacterial Biodiversity-Ecosystem Functioning Relations Are Modified by Environmental Complexity. PLoS ONE 2010;5:e10834
Larsson, D.G.J. Pollution from drug manufacturing: review and perspectives. Philosophical Transactions of the Royal Society B: Biological Sciences 2014;369
Larsson, D.G.J.; de Pedro, C.; Paxeus, N. Effluent from drug manufactures contains extremely high levels of pharmaceuticals. Journal of Hazardous Materials 2007;148:751-755
Laxminarayan, R.; Duse, A.; Wattal, C.; Zaidi, A.K.M.; Wertheim, H.F.L.; Sumpradit, N.; Vlieghe, E.; Hara, G.L.; Gould, I.M.; Goossens, H.; Greko, C.; So, A.D.; Bigdeli, M.; Tomson, G.; Woodhouse, W.; Ombaka, E.; Peralta, A.Q.; Qamar, F.N.; Mir, F.; Kariuki, S.; Bhutta, Z.A.; Coates, A.; Bergstrom, R.; Wright, G.D.; Brown, E.D.; Cars, O. Antibiotic resistance - the need for global solutions. The Lancet Infectious Diseases 2013;13:1057-1098
Li, D.; Yang, M.; Hu, J.; Ren, L.; Zhang, Y.; Li, K. Determination and fate of oxytetracycline and related compounds in oxytetracycline production wastewater and the receiving river. Environmental Toxicology and Chemistry 2008;27:80-86
Liu, Y.; Zhang, J.; Gao, B.; Feng, S. Combined effects of two antibiotic contaminants on Microcystis aeruginosa. Journal of hazardous materials 2014;279:148-155
Lundström, S.V.; Östman, M.; Bengtsson-Palme, J.; Rutgersson, C.; Thoudal, M.; Sircar, T.; Blanck, H.; Eriksson, K.M.; Tysklind, M.; Flach, C.-F.; Larsson, D.G.J. Minimal selective concentrations of tetracycline in complex aquatic bacterial biofilms. Science of The Total Environment 2016;553:587-595
Miao, X.-S.; Bishay, F.; Chen, M.; Metcalfe, C.D. Occurrence of Antimicrobials in the Final Effluents of Wastewater Treatment Plants in Canada. Environmental Science & Technology 2004;38:3533-3541
Moermond, C.T.A.; Kase, R.; Korkaric, M.; Ågerstrand, M. CRED: Criteria for reporting and evaluating ecotoxicity data. Environmental Toxicology and Chemistry 2016;35:1297-1309

Monteiro, S.C.; Boxall, A.B.A. Occurrence and Fate of Human Pharmaceuticals in the Environment. in: Whitacre D.M., ed. Reviews of Environmental Contamination and Toxicology, Vol 202. New York: Springer; 2010
Moriyama, T.; Sato, N. Enzymes involved in organellar DNA replication in photosynthetic eukaryotes. Frontiers in Plant Science 2014;5
Näslund, J.; Hedman, J.E.; Agestrand, C. Effects of the antibiotic ciprofloxacin on the bacterial community structure and degradation of pyrene in marine sediment. Aquatic Toxicology 2008;90:223-227
Negri, M.-C.; Lipsitch, M.; Blázquez, J.; Levin, B.R.; Baquero, F. Concentration-Dependent Selection of Small Phenotypic Differences in TEM β-Lactamase-Mediated Antibiotic Resistance. Antimicrobial Agents and Chemotherapy 2000;44:2485-2491
O'Neill, J. Antimicrobial Resistance:Tackling a crisis for the health and wealth of nations. London: Wellcome Trust 2014;
O'Neill, J. Antimicrobials in agriculture and the environment: reducing unnecessary use and waste. London: Wellcome Trust 2015;
O'Neill, J. Securing new drugs for future generations: the pipeline of antibiotics. London: Wellcome Trust 2015;
OECD. Test No. 209: Activated Sludge, Respiration Inhibition Test (Carbon and Ammonium Oxidation) ed^eds: OECD Publishing; 2010
OECD. Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test, OECD Guidelines for the Testing of Chemicals, Section 2. Paris: OECD Publishing; 2011
OECD. Test No. 211: Daphnia magna Reproduction Test ed^eds: OECD Publishing; 2012
Pruden, A.; Larsson, D.J.; Amézquita, A.; Collignon, P.; Brandt, K.K.; Graham, D.W.; Lazorchak, J.M.; Suzuki, S.; Silley, P.; Snape, J.R. Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. Environmental Health Perspectives (Online) 2013;121:878
Quinlan, E.L.; Nietch, C.T.; Blocksom, K.; Lazorchak, J.M.; Batt, A.L.; Griffiths, R.; Klemm, D.J. Temporal Dynamics of Periphyton Exposed to Tetracycline in Stream Mesocosms. Environmental Science & Technology 2011;45:10684-10690
Santos, R.; Ursu, O.; Gaulton, A.; Bento, A.P.; Donadi, R.S.; Bologa, C.G.; Karlsson, A.; Al-Lazikani, B.; Hersey, A.; Oprea, T.I.; Overington, J.P. A comprehensive map of molecular drug targets. Nat Rev Drug Discov 2017;16:19-34
Schaefer, A.M.; Goldstein, J.D.; Reif, J.S.; Fair, P.A.; Bossart, G.D. Antibiotic-Resistant Organisms Cultured from Atlantic Bottlenose Dolphins (Tursiops truncatus) Inhabiting Estuarine Waters of Charleston, SC and Indian River Lagoon, FL. EcoHealth 2009;6:33-41
Scholz, S.; Sela, E.; Blaha, L.; Braunbeck, T.; Galay-Burgos, M.; Garcia-Franco, M.; Guinea, J.; Kluver, N.; Schirmer, K.; Tanneberger, K.; Tobor-Kaplon, M.; Witters, H.; Belanger, S.; Benfenati, E.; Creton, S.; Cronin, M.T.D.; Eggen, R.I.L.; Embry, M.; Ekman, D.; Gourmelon, A.; Halder, M.; Hardy, B.; Hartung, T.; Hubesch, B.; Jungmann, D.; Lampi, M.A.; Lee, L.; Leonard, M.; Kuster, E.; Lillicrap, A.; Luckenbach, T.; Murk, A.J.; Navas, J.M.; Peijnenburg, W.; Repetto, G.; Salinas, E.; Schuurmann, G.; Spielmann, H.; Tollefsen, K.E.; Walter-Rohde, S.; Whale, G.; Wheeler, J.R.; Winter, M.J. A

European perspective on alternatives to animal testing for environmental hazard identification and risk assessment. Regulatory Toxicology and Pharmacology 2013;67:506-530
Shih, P.M.; Wu, D.; Latifi, A.; Axen, S.D.; Fewer, D.P.; Talla, E.; Calteau, A.; Cai, F.; Tandeau de Marsac, N.; Rippka, R.; Herdman, M.; Sivonen, K.; Coursin, T.; Laurent, T.; Goodwin, L.; Nolan, M.; Davenport, K.W.; Han, C.S.; Rubin, E.M.; Eisen, J.A.; Woyke, T.; Gugger, M.; Kerfeld, C.A. Improving the coverage of the cyanobacterial phylum using diversity-driven genome sequencing. Proceedings of the National Academy of Sciences 2013;110:1053-1058
Singer, A.C.; Colizza, V.; Schmitt, H.; Andrews, J.; Balcan, D.; Huang, W.E.; Keller, V.D.; Vespignani, A.; Williams, R.J. Assessing the ecotoxicologic hazards of a pandemic influenza medical response. Environmental health perspectives 2011;119:1084
Smith, R.D.; Coast, J. Antimicrobial resistance: a global response. Bulletin of the World Health Organization 2002;80:126-133
Taylor, N.G.H.; Verner-Jeffreys, D.W.; Baker-Austin, C. Aquatic systems: maintaining, mixing and mobilising antimicrobial resistance? Trends in Ecology & Evolution 2011;26:278-284
US Food and Drug Administration. Guidance for industry, environmental assessment of human drug and biologics applications. Washington (DC): FDA 1998;
Van Boeckel, T.P.; Gandra, S.; Ashok, A.; Caudron, Q.; Grenfell, B.T.; Levin, S.A.; Laxminarayan, R. Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data. The Lancet Infectious Diseases 2014;14:742-750
Vestel, J.; Caldwell, D.J.; Constantine, L.; D'Aco, V.J.; Davidson, T.; Dolan, D.G.; Millard, S.P.; Murray-Smith, R.; Parke, N.J.; Ryan, J.J.; Straub, J.O.; Wilson, P. Use of acute and chronic ecotoxicity data in environmental risk assessment of pharmaceuticals. Environmental Toxicology and Chemistry 2015:n/a-n/a
Wall, M.K.; Mitchenall, L.A.; Maxwell, A. Arabidopsis thaliana DNA gyrase is targeted to chloroplasts and mitochondria. Proceedings of the National Academy of Sciences of the United States of America 2004;101:7821-7826
Watkinson, A.J.; Murby, E.J.; Kolpin, D.W.; Costanzo, S.D. The occurrence of antibiotics in an urban watershed: From wastewater to drinking water. Science of The Total Environment 2009;407:2711-2723
Webster, L.F.; Thompson, B.C.; Fulton, M.H.; Chestnut, D.E.; Van Dolah, R.F.; Leight, A.K.; Scott, G.I. Identification of sources of Escherichia coli in South Carolina estuaries using antibiotic resistance analysis. Journal of Experimental Marine Biology and Ecology 2004;298:179-195
Whitton, B.A. Ecology of Cyanobacteria II: Their Diversity in Space and Time ed^eds: Springer Netherlands; 2012
World Health Organization. Antimicrobial resistance: 2014 global report on surveillance ed^eds: World Health Organization; 2014
Zhang, H.; Deng, X.; Miki, D.; Cutler, S.; La, H.; Hou, Y.-J.; Oh, J.; Zhu, J.-K. Sulfamethazine Suppresses Epigenetic Silencing in Arabidopsis by Impairing Folate Synthesis. The Plant Cell 2012;24:1230-1241

1405 Zheng, S.; Qiu, X.; Chen, B.; Yu, X.; Liu, Z.; Zhong, G.; Li, H.; Chen, M.; Sun, G.; Huang,
1406 H.; Yu, W.; Freestone, D. Antibiotics pollution in Jiulong River estuary:
1407 Source, distribution and bacterial resistance. Chemosphere
1408 2011;84:1677-1685
1409 Zinsstag, J.; Schelling, E.; Waltner-Toews, D.; Tanner, M. From "one medicine" to
1410 "one health" and systemic approaches to health and well-being.
1411 Preventive Veterinary Medicine 2011;101:148-156
1412 Zou, S.; Xu, W.; Zhang, R.; Tang, J.; Chen, Y.; Zhang, G. Occurrence and distribution
1413 of antibiotics in coastal water of the Bohai Bay, China: Impacts of river
1414 discharge and aquaculture activities. Environmental Pollution
1415 2011;159:2913-2920
1416